Pharmacological Research 75 (2013) 15–27

Contents lists available at SciVerse ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Invited review

MicroRNAs as pharmacological targets in endothelial cell function and dysfunction

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ARTICLE INFO

Article history:
Received 24 January 2013
Received in revised form 3 April 2013
Accepted 5 April 2013

Keywords:
Endothelial cells
MicroRNAs
Vascular disease
MicroRNA therapeutics
Endothelial cell dysfunction

ABSTRACT

Endothelial cell dysfunction is a term which implies the dysregulation of normal endothelial cell functions, including impairment of the barrier functions, control of vascular tone, disturbance of proliferative, migratory and morphogenic capacities of endothelial cells, as well as control of leukocyte trafficking. MicroRNAs are short non-coding RNAs that have emerged as critical regulators of gene expression acting predominantly at the post-transcriptional level. This review summarizes the latest insights in the identification of endothelial-specific microRNAs and their targets, as well as their roles in controlling endothelial cell functions in both autocrine and paracrine manner. In addition, we discuss the therapeutic potential for the treatment of endothelial cell dysfunction and associated vascular pathophysiological conditions.

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1. Introduction

The endothelium is the monolayer of endothelial cells (ECs) lining the lumen of blood vessels in every organ system. These cells function as a protective biocompatible barrier between all tissues and the circulating blood [1]. ECs facilitate the bidirectional passage of nutrient substances and active molecules from blood to tissues, but also play a major role in controlling the passage of blood cells themselves. ECs are specially designed and spatially located to detect changes in hemodynamic forces and blood-borne signals.

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http://dx.doi.org/10.1016/j.phrs.2013.04.002

In addition, ECs regulate the release of a number of autocrine and paracrine factors in response to these signals to favor the maintenance of vascular homeostasis [2–9]. Therefore, normal EC function is critical for all aspects of vascular homeostasis (*i.e.* control of blood vessel development, growth and differentiation; control of leukocyte trafficking; control of vascular tone; control of vascular barrier; control of platelet function, coagulation and fibrinolysis). These have been reviewed in depth elsewhere [2–4,6–8,10].

EC dysfunction disrupts the balance between vasoconstriction and vasodilation and initiates a number of events that trigger EC activation and predispose the vessel wall to increased endothelial permeability, leukocyte adherence, endothelial proliferation, pro-oxidation and thrombosis [5,7,11–18]. Perturbations in EC functions have been implicated in several diseases including atherosclerosis, diabetes, tumor metastasis, inflammatory diseases (*e.g.* rheumatoid arthritis) and hypertension [5,12,16,19–21]. Importantly, the processes involved in EC activation require integration of the molecular and cellular events induced by both stimulatory and inhibitory signals. These include signal transduction pathways leading to transcriptional regulation of gene expression programs, as well as post-transcriptional and post-translational modifications that fine-tune this response. In this regard, microRNAs (miRNAs) have emerged as critical regulators of gene expression, acting predominantly at the post-transcriptional level [22–25].

In the present review we summarize the latest insights in the identification of endothelial-specific miRNAs and their targets, as well as their role in regulating EC functions both in an autocrine and paracrine fashion. In addition, we discuss their therapeutic potential in the treatment of EC dysfunction and associated vascular pathophysiological conditions.

2. MicroRNA biogenesis and function

MiRNAs are short noncoding RNAs that have emerged as critical regulators of gene expression acting predominantly at the post-transcriptional level [23,25]. According to the miRNA.org data resource, as of April 2013, 1100 miRNAs have been annotated in *Homo sapiens* and 717 in *Mus musculus* [26], while MirBase reports 2042 human and 1281 murine miRNAs [27,28]. Canonically these small RNAs are transcribed by RNA polymerase II from individual miRNA genes, introns of protein coding genes, or polycistronic transcripts as capped and polyadenylated primary miRNA transcripts (pri-miRNA) [29,30]. Then the pri-miRNA is processed in the nucleus into a ~70-nucleotide precursor hairpin (pre-miRNA) by ribonuclease III (RNase III), called Drosha in cooperation with a dsRNA binding protein, DiGeorge syndrome critical region gene 8 (DGCR8) [31,32]. Additionally, there is a group of intronic miRNAs (miRtrons) that bypass the Drosha pathway and are produced by splicing and debranching [33]. The transport receptor Exportin-5, RanGTP-dependent dsRNA-binding protein, exports the pre-miRNAs to the cytoplasm [34], where they are further processed by the RNaseIII enzyme, Dicer, into an approximately 22 nt miRNA/miRNA* duplex [35,36]. Although the miRNA/miRNA* duplex are produced in equal amounts by transcription, the abundance of mature strands is asymmetric at the steady state and it depends on the thermodynamic stability of the 5′ end of the miRNA strand [37,38]. Usually the miRNA* strand (or passenger strand) is the least thermodynamically stable and is rapidly degraded, however recent reports have shown that it has well-conserved target recognition sites that are also functional [39,40].

Following processing, the selected miRNAs are incorporated into the RNA-induced silencing complex (RISC) [41,42] which mediates the miRNA binding to the 3′ untranslated regions (3′UTR) of target messenger mRNA (mRNA) and negatively regulates gene expression by translational inhibition or target mRNA degradation or a combination of both [41,43,44]. In order to repress the transcript, it is crucial that the nucleotides in position 2–8 of the miRNA (called the seed sequence) are almost perfectly complimentary to regions at the 3′UTR of their target genes [45].

Computational predictions have revealed that 60% of protein-coding genes harbor miRNA target sites in their 3′UTR [46] and that a single miRNA can modulate the expression of hundreds genes. Not only are certain miRNA genes highly conserved in animals, but their target sites in the 3′UTR of genes are also under positive evolutionary selection. MiRNAs are powerful modulators of genetic networks and they do so by acting both in a coherent and incoherent fashion on their target genes [47,48]. MiRNAs, rather than functioning as regulatory on-off switches, often function to modulate or fine-tune cellular phenotypes [24,49,50]. The deregulation of miRNA expression could affect multiple cellular processes. Therefore it is not surprising that miRNAs have been implicated in various processes, from development to aging [24,49,50]. Additionally, many miRNAs exhibit striking tissue specific expression patterns [51–56], suggesting a cell type-specific function [51].

MiRNAs are necessary for development and organogenesis. Loss of Dicer in mice results in embryonic lethality at E7.5 and embryonic stem cells cannot be obtained from Dicer null mutants, suggesting that Dicer is especially important in maintaining the pluripotent status [57,58]. Also Ago loss gives rise to severe developmental defects by E10.5 [59]. At E11.5–E14.5 the developing mouse embryo expresses Dicer1 and the Ago proteins only in certain anatomical compartments and in a dynamic fashion, suggesting a further role for miRNAs in organogenesis as well [60]. Given the essential role of the miRNA processing machinery in development, it is not surprising that miRNAs have been shown to be relevant in vascular development [51] and vascular functions [61–67].

3. Control of blood vessel development, growth and differentiation by endothelial microRNAs

The *de novo* generation and remodeling of blood vessels is essential for embryonic growth and throughout postnatal life. During adulthood, the endothelium remains essentially quiescent to fulfill its main function in conducting nutritive blood flow to organs, with turnover rates on the orders of months to years. Rapid changes in EC proliferation rates occur following activation of the endothelium by angiogenic cytokines [68–71]. In fact, in the healthy adult, angiogenesis occurs only in select phases of the female reproductive cycle and as a protection mechanism in wound healing/tissue repair and is almost exclusively associated with pathology when angiogenesis is induced by micro-environmental factors such as hypoxia or inflammation [68,70,72–75]. ECs play a key role in angiogenesis which is dependent on the proliferation, migration and differentiation of these cells [76].

The pathological processes associated with angiogenesis include diseases as diverse as cancer, macular degeneration, psoriasis, diabetic retinopathy, thrombosis, and inflammatory disorders, including arthritis and atherosclerosis. Moreover, insufficient angiogenesis is characteristic of ischemic heart disease, peripheral vascular disease and pre-clampsia [68,69]. The above examples represent the broad array of diseases that are associated with dysfunction of the angiogenic activated EC phenotype.

The first evidence of the importance of miRNAs in vascular development was shown by Yang et al. who generated a Dicer ex$^{1/2}$ knockout mice, where Dicer was hypomorphic because of the deletion of its first and second exons. However, homozygous embryos

died between days E12.5 and E14.5 of impaired vascular formation and maintenance, showing that Dicer was essential for normal vascular development [51]. Interestingly, the expression of vascular endothelial growth factor (VEGF) and the VEGF receptors, FLT1 and VEGF receptor 2 (VEGFR2), was upregulated in mutant embryos while the expression of the angiopoietin receptor, Tie-1, was lower than wild type embryos, suggesting that the impaired angiogenesis was due to the deregulation of these critical angiogenic regulators [51]. In the context of ECs, the knockdown of Dicer *in vitro* [77] indicated that miRNAs were important for regulating the angiogenic functions of ECs. In fact, the silencing of Dicer modulated the expression of several proteins implicated in the control of vascular tone and angiogenesis (*e.g.* VEGFR2, TEK/Tie-2, interleukin 8 (IL)-8 and endothelial nitric oxide synthase (eNOS) among others) and impaired EC proliferation and cord formation. Later Kuehbacher et al. showed that the silencing of either Drosha or Dicer negatively regulated the expression of miR-let7a and miR-27b while increased the anti-angiogenic factor thrombospondin-1 (Tsp-1) [78]. In addition, the knockdown of Drosha and Dicer reduced sprout formation [78]. Finally, these results were confirmed *in vivo* using EC-specific Dicer knockout mice [79]. Although the inactivation of Dicer in the endothelium did not alter vascular development, it reduced the postnatal angiogenic response to multiple stimuli such as exogenous VEGF or in models of tumorigenesis, limb ischemia and wound healing [79].

The aforementioned experimental approaches reveal the consequences of a block in miRNA biogenesis. However, the majority of these studies do not provide information regarding the functions of specific miRNAs. Examples that illustrate the roles of individual miRNAs in blood vessel development, growth and differentiation are presented below and depicted in Fig. 1A.

MiR-126 is considered the prototype of an endothelial-specific miRNA. It is highly expressed in vascularized tissues, ECs and hematopoietic stem cells [79–84]. Interestingly, the miR-126 gene is located within an intron of the epidermal growth factor-like-domain 7 (EGFL7) gene, also known as Vascular Endothelial-statin (VE-statin), which is mostly expressed in ECs and involved in vascular tubulogenesis [85,86]. In contrast, the host gene EGFL7 is modestly expressed in mature vessels in normal adult tissue and highly expressed in tumor endothelium [85,86]. The role of miR-126 in vascular integrity and angiogenesis was reported by targeted deletion of miR-126 in mice [87,88] and its knockdown in zebrafish [89]. MiR-126 was shown to target sprouty-related protein (SPRED1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-β), both negative regulators of the VEGF pathway [87–89]. Additionally, miR-126 also enhanced Angiopoietin-1 (Ang-1) signaling through PIK3R2/p85-β repression, implicating that miR-126 is also involved in vessel stabilization and maturation [90]. The miR-126/Egfl7 gene is transcriptionally regulated by Ets-1 and Ets-2 in ECs [82]. Recently, Nicoli et al. described a new mechanism for the regulation of miR-126 by blood flow in zebrafish. In particular, blood flow, through the mechanosensitive transcription factor kruppel-like factor 2a (KLF2a), induced miR-126 expression, which in turn activated the VEGF pathway [91]. Consistent with the

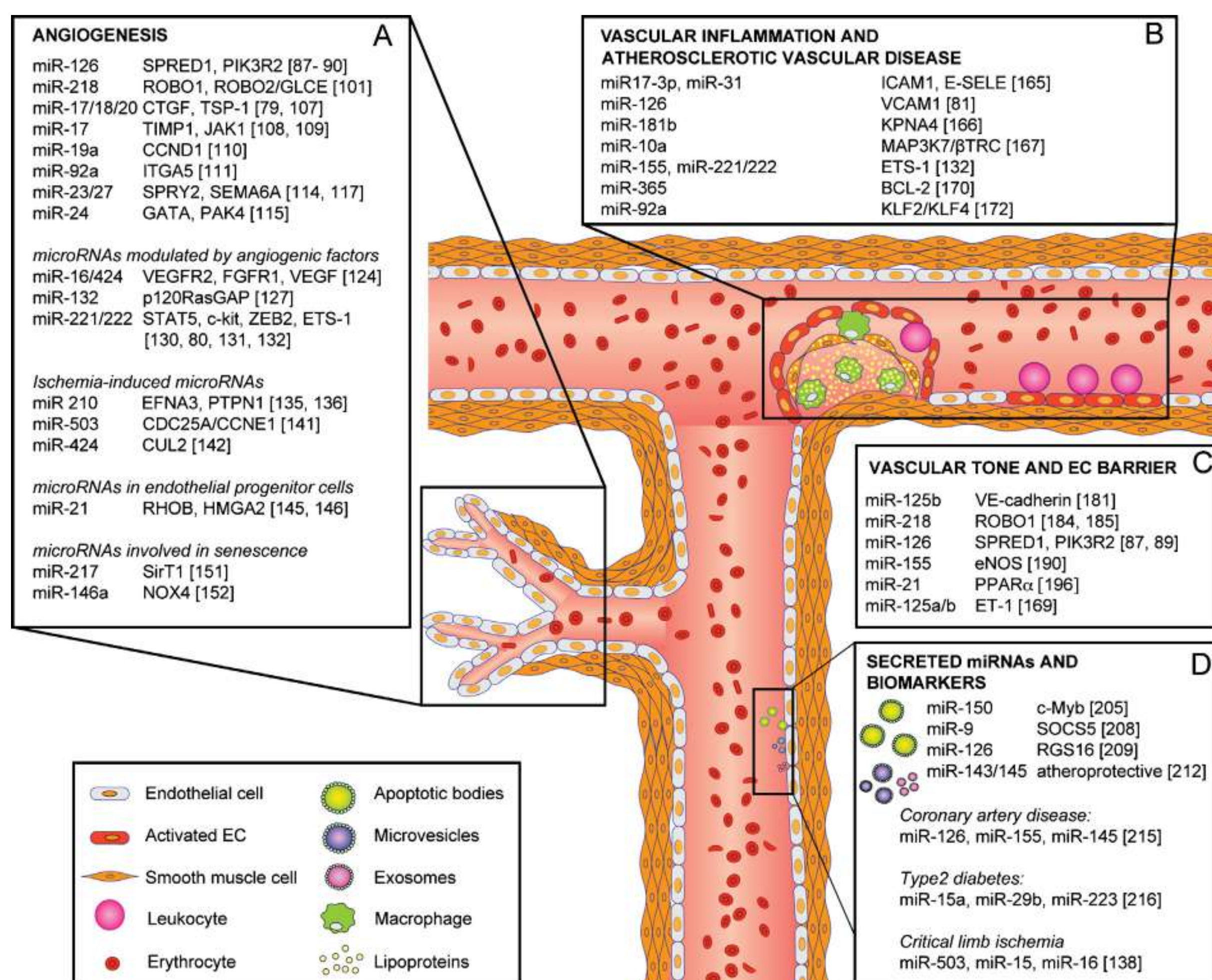

Fig. 1. Role of miRNAs in endothelial cell phenotype, functions and vascular disease and their direct targets. MicroRNAs involved in: (A) angiogenesis, (B) vascular inflammation and atherosclerotic vascular disease, (C) vascular tone and endothelial cell barrier, and (D) secreted microRNAs and biomarkers.

angiogenic properties of miR-126, Van Solingen et al. demonstrated that the use of antagomiR-126 impairs ischemia-induced angiogenesis [92]. Interestingly, the expression of miR-126 has been shown to be downregulated in different cancers acting as a tumor suppressor by inhibiting tumor growth [93–97]. Additionally, miR-126 repressed VEGF expression in lung cancer cells [98]. However, it should be considered that miR-126 regulates the EC response to proangiogenic factors (*e.g.* VEGF and Ang-1) thus promoting angiogenesis. Therefore, further analysis to elucidate the role of miR-126 in tumor vasculature is needed to ascertain whether miR-126 could be a therapeutic tool to target tumor growth and angiogenesis.

As noted above, the relationship between miRNAs and their host gene is especially relevant in miRNAs encoded within an angiogenic host gene. The highly conserved miR-218 is an intronic miRNA encoded within the Slit2 and Slit3 genes [99–101]. Slits are secreted glycoproteins, which are the main ligands for Roundabout receptors (Robos). Slit/Robo signaling is mediated by heparan sulfate proteoglicans (HSPG), and regulates axon and vascular guidance. Small et al. reported the relevance of miR-218 in the regulation of Slit/Robo signaling through repression of Robo1, Robo2 and glucuronyl C5-epimerase (GLCE), an enzyme involved in HSPG biosynthesis [101]. Importantly, the silencing of miR-218 resulted in abnormal EC migration and reduced complexity of the retinal vasculature [101]. Recently, Fish et al. reported that Slt2-Robo1-miR-218 signaling is also required during heart development in zebrafish [99].

The miR-17-92 cluster is one of the best-characterized polycistronic miRNAs and is located in intron 3 of the C13orf25 gene (chromosome 13q31.3). MiR-17-92 encodes 6 individual miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a, which are tightly grouped within an 800 base-pair region [102,103]. The miR-17-92 cluster is involved in cell proliferation, suppresses apoptosis of cancer cells, and induces tumor angiogenesis. The first evidence of the oncogenic role of miR-17-92 was suggested by the amplification of the genomic location corresponding to miR-17-92 in lymphoma and solid tumors [102,104]. MiR-17-92 is regulated at the transcriptional level by the oncogenic transcription factor, c-Myc and its downstream effector E2F that promotes cell cycle progression [105]. MiR-17 and miR-20, which have identical seed sequences, repress E2F1, providing a tight regulation of c-Myc-mediated cellular proliferation. In addition, the miR-17-92 cluster is regulated by the c-Myc homologue MYCN in neuroblastoma, and regulates cell cycle progression and apoptosis through repressing p21 and Bcl-2 interacting mediator of cell death (BIM) [106]. Overexpression of the entire miR-17-92 cluster in Myc-induced tumors increases angiogenesis by a paracrine mechanism [107]. This pro-angiogenic function has been attributed to the downregulation of anti-angiogenic proteins such as connective tissue growth factor (CTGF) and Tsp-1, which are targeted by miR-18 and miR-19 [79,107]. In addition, miR-17 also represses the anti-angiogenic factor tissue inhibitor of metalloproteinase 1 (TIMP1) [108]. These findings were also confirmed in the context of ECs, where the combined inhibition of miR-17, miR-18 and miR-20a reduced proliferation and their overexpression rescued the decreased morphogenic capacity of ECs induced by Dicer silencing [79]. Interestingly, these components of the miR-17-92 cluster (miR-17, miR-18 and miR-20a) were induced upon VEGF treatment [79], therefore indicating that these miRNAs contribute to promote VEGF-induced angiogenic responses. However, in another model system, the overexpression of different components of miR-17-92, in particular miR-17, -18a, -19a, and -20a, impaired sprouting whereas their inhibition increased sprouting in ECs [109]. In this context, miR-17 was shown to negatively regulate the expression of the proangiogenic Janus kinase 1 (JAK1) [109], while miR-19a was shown to modulate cell cycle progression by targeting cyclin D1 (CCND1) in ECs [110]. Additionally, overexpression of the remaining member of the cluster, miR-92a, decreased sprouting *in vitro* and also induced defects in intersegmental vessel formation in zebrafish [111]. Interestingly, miR-92a overexpression reduced the expression of integrin subunits $\alpha5$ (ITG$\alpha5$) and $\alpha\nu$ (ITG$\alpha\nu$), of the sphingosine-1-phosphate receptor 1 (S1P1) and of mitogen-activated kinase kinase 4 (MKK4) and eNOS in ECs [111].

The miR-106b-25 cluster, which is evolutionarily related to the miR-17-92 cluster, is also involved in angiogenesis. A recent paper from Semo et al. [112] revealed that absence of the cluster in a model of hindlimb ischemia impaired the capacity of restoring normal blood flow and significantly decreased capillary formation. This effect was attributed to the ability of the cluster to inhibit proliferation, migration and viability in ECs. However, the targets of miR-106b-25 responsible for this phenotype were not investigated.

The miR-23-24-27 cluster is expressed in ECs and vascularized tissues and is heavily involved in angiogenesis There are two miR-23-24-27 genes in mammals, an intergenic miR-23a–27a–24-2 cluster (chromosome 9q22) and an intronic 23b–27b–24-1 cluster (chromosome 19p13) [113]. This cluster mediates diverse process, such as proliferation, cell cycle arrest, apoptosis and migration [113]. Moreover, miR-23-24-27 has been involved in the regulation of angiogenesis during vascular disorders and ischemic heart disease [114,115], and their role has been elucidated *in vitro* and *in vivo* [116]. In particular, miR-23 and miR-27 repress Sprouty2 (SPRY2) and Semaphorin-6A (SEMA6A), which negatively regulate Ras/MAPK signaling and VEGFR2 mediated signaling, respectively [114]. In agreement with these data, Urbich et al. showed that the inhibition of miR-27a and miR-27b impairs sprouting in ECs *in vitro* and embryonic vessel formation in zebrafish through repression of SEMA6A [117]. Importantly, in a murine model of laser-induced choroidal neovascularization, the administration of locked nucleic acid (LNA)-anti-miR-23/27 increased the expression of SPRY2 and SEMA6A and impaired retinal vascular development [114]. The other member of the cluster, miR-24, is highly expressed in cardiac ECs and its expression increases in cardiac ischemia and in hypoxic conditions [115]. MiR-24 overexpression enhanced endothelial apoptosis and impaired angiogenesis by targeting the transcription factor GATA2 and the p21-activated kinase (PAK4). MiR-24 overexpression, as well as knockdown of miR-24 targets, hindered vascular development in zebrafish, whereas endogenous inhibition of miR-24 by antagomirs enhanced angiogenesis and cardiac function after myocardial infartion in mice [115]. Interestingly, the expression of several members of the miR-23-24-27 cluster, in particular miR-23b and miR-27, are upregulated by shear stress and correlate with pulsatile shear (PS)-induced EC growth arrest. Furthermore, the inhibition of miR-23b using antagomirs partially rescued PS-induced growth arrest by an increase in the phosphorylation of the retinoblastoma (Rb) protein [118].

3.1. MicroRNAs regulated by angiogenic factors: extinguishers or igniters?

A fine balance between positive and negative regulators controls angiogenesis [68,72,75,119]. While there are many angiogenic inducers, VEGF and basic fibroblast growth factors (bFGF) are probably the most critical and potent ones. The pro-angiogenic effect of VEGF and bFGF is mediated through the VEGFR2, which is selectively expressed in vascular ECs, or the FGF receptor 1 (FGFR1), respectively [72,120–123]. Activation of these receptors stimulates the angiogenic cascade that leads to the degradation of the extracellular matrix, migration to the perivascular space, proliferation and formation of tubes [71,76]. MiRNAs do not only control the angiogenic response, but their expression may also be regulated by pro-angiogenic factors (*e.g.* as introduced above for the miR-17-92 cluster whose expression is regulated by VEGF to promote the angiogenic response of ECs [79]). Some examples of

microRNAs regulated by angiogenic factors can be found below and are illustrated in Fig. 1A.

MiR-16 and miR-424 have been described to be anti-angiogenic miRNAs because of their capability to repress VEGFR2, FGFR1 and VEGF in ECs [124]. Specifically, miR-16 or miR-424 overexpression reduces EC proliferation, migration, and impairs cord formation on matrigel *in vitro*. Conversely, the endogenous inhibition of miR-16 and miR-424 had opposite effect on these phenotypes. Importantly, lentiviral overexpression of miR-16 reduced blood vessel formation *in vivo*. Interestingly, miR-16 and miR-424 are intriguing examples of miRNAs whose expression is regulated by proangiogenic factors such as VEGF and bFGF at the transcriptional level [124]. Given their negative effects on angiogenesis, their transcriptional regulation upon angiogenic stimuli provides a negative feedback control of angiogenesis [124].

Another example of a miRNA regulated by pro-angiogenic factors that conversely has an angiogenic phenotype is miR-132. The endothelial miR-132 is an intergenic miRNA (chromosome 17) and its expression is regulated by the transcription factor cAMP response element binding protein (CREB) [125,126]. Annand et al. showed that miR-132 acts as a switch to induce neovascularization by suppressing endothelial p120RasGAP expression, a GTPase activating protein that attenuates p21 Ras activity [127]. MiR-132 expression levels are undetectable in quiescent endothelium while it is highly expressed in endothelium in tumors and hemangiomas [127]. Several pro-angiogenic factors, such as VEGF and bFGF, increased the expression of miR-132 through CREB activation in ECs [127]. Overexpression of miR-132 increased proliferation and cord formation in ECs *in vitro*, whereas anti-miR-132 produced a converse effect that was also observed *in vivo* by reducing both developmental and pathological angiogenesis [127].

Interestingly, Dentelli et al. showed that interleukin 3 (IL)-3 and bFGF negatively modulated the expression of miR-221/miR-222 in ECs. Several studies have demonstrated that IL-3 and bFGF are released by infiltrated T-lymphocytes in the atherosclerotic plaque, promoting neovascularization [128,129]. STAT5A, a transcription factor that regulates the expression of genes involved in cell proliferation and migration [128], was identified as target for miR-222 [130]. Interestingly, upregulation of STAT5A due to IL-3/bFGF-induced down-regulation of miR-222 controled EC proliferation and migration and therefore facilitated intraplaque neovascularization during atherosclerosis. Moreover, in advanced lesions the authors found an increased proliferation rate of ECs lining vessels, which correlated with a diminished expression of miR-222 [130]. In line with these findings, Poliseno et al. previously reported that miR-221/miR-222 regulated proliferation and migration through the regulation of c-kit in ECs [80]. On the other hand, miR-221 has been shown to inhibit angiogenesis by targeting zinc finger E-box binding homeobox 2 (ZEB2), a modulator of the epithelial-mesenchymal transition, leading to decreased EC proliferation [131]. Interestingly, miR-221/miR-222 repressed the expression of the transcription factor Ets-1, which regulates genes involved in angiogenesis, inflammation and vascular remodeling [132].

3.2. Ischemia and microRNAs

The formation of new blood vessels is an essential component in pathologies such as ischemia, where interrupted blood flow deprives tissues of oxygen and nutrients necessary to maintain their normal functions. In ischemia, lack of oxygen, or hypoxia, is associated with angiogenic stimuli (like expression of VEGF) in an attempt for the cell to maintain tissue homeostasis [133]. In response to ischemia, not only the expression of certain miRNAs is increased, but their upregulation also modulates critical events associated with ischemia-induced angiogenesis (Fig. 1A).

MiR-210, also referred to as hypoxamir, is an intronic miRNA contained within the sequence of the non-protein transcript MIR210HG located on chromosome 11 [134] and its expression is induced in hypoxic conditions in a hypoxia inducible factor-1α (HIF-1α)-dependent manner. Fasanaro et al. showed that hypoxia-induced miR-210 enhanced the development of capillary-like structures and VEGF-induced chemotaxis in ECs [135]. Interestingly, Hu et al. showed that miR-210 promoted angiogenesis and inhibited apoptosis while improving cardiac function in a murine model of myocardial infarction, suggesting that miR-210 might be a promising therapy for ischemic disease [136]. This miRNA exerts its pro-angiogenic and anti-apoptotic effects by downregulating ephrinA3 (EFNA3) [135,136] and protein tyrosine phosphatase 1b (PTPN1) [136]. Additionally, the expression of miR-210 is upregulated in atherosclerotic plaques providing a link between this miRNA and EC dysfunction in atherosclerosis [137].

Consistent with the notion of miR-16 being upregulated in response to VEGF, Spinetti et al. reported that patients with critical limb ischemia showed higher levels of miR-16 and miR-15a in proangiogenic circulating cells (PACs), which impair PACs functions [138]. Moreover, Hullinger et al. showed that miR-15 is an important regulator in cardiac ischemic injury using LNA-anti-miR-15 in mice [139]. Notably, targeting of miR-15 reduced infarct size, inhibited cardiac remodeling and enhanced cardiac function in response to ischemic damage, proving that miR-15 might be an interesting potential therapeutic target [139]. Recently, Yin et al. reported that the nuclear receptor, peroxisome proliferator-activated receptor δ (PPARδ) binds directly to the PPRE site in the miR-15 promoter region, repressing its expression [140]. Interestingly, the levels of PPARδ decreased upon oxygen-glucose deprivation in cerebral vascular ECs, an ischemia like insult, increasing EC apoptosis as well as necrotic cell death. In contrast, the activation of PPARδ inhibited the levels of miR-15 and subsequently, increased Bcl2 protein levels resulting in a reduction of caspase 3 activity, of Golgi fragmentation and of cell death in mouse cerebral endothelium after ischemic insult *in vitro* and *in vivo* [140]. Targeting miR-15 or pharmacological activation of PPARδ may be a promising therapeutic option to induce apoptosis in stroke-induced vascular damage.

A recent study on miR-503, whose seed region differs in a nucleotide from the canonical seed sequence of the miR-16 family, showed that the miRNA is upregulated in culture conditions mimicking diabetes mellitus and ischemia-associated starvation in ECs [141]. MiR-503 decreased EC proliferation, migration and cord formation by targeting cell division cycle 25 (CDC25A) and cyclin E1 (CCNE1). Furthermore, administration of anti-miR-503 improved blood flow recovery and angiogenesis in diabetic mice with limb ischemia. All these data suggest that the regulation of miR-503 levels could be a therapeutic tool in diabetic patients [141].

Given the complex stimuli in hypoxia, the angiogenic function of certain miRNAs differs compared to normoxia. For instance, miR-424 promotes angiogenesis in hypoxic conditions [142]. Specifically, hypoxia increases the expression of miR-424 by PU.1-dependent transactivation in ECs. MiR-424 was shown to target cullin 2 (CUL2), a scaffolding protein critical for the assembly of the ubiquitin ligase system, which stabilizes HIF-1α resulting in increased proliferation and migratory capabilities in ECs [142].

3.3. Endothelial progenitor cells and microRNAs

Improvement of neovascularization is a therapeutic option to rescue tissue from critical ischemia [143]. In this regard, circulating endothelial progenitor cells (EPCs) have been suggested to play an important role in maintenance of vascular integrity. Recent work also indicates that miRNAs play a critical role in EPC biology (Fig. 1A). Indeed, Minami et al. showed an increase of

miR-221/miR-222 in circulating EPCs isolated from patients with coronary artery disease (CAD) or ischemic heart disease. Importantly, the expression levels of miR-221/miR-222 and number of EPCs were negatively correlated in CAD patients. Notably, the expression of miR-221/miR-222 was reduced in EPCs isolated from CAD patients receiving long-term statin therapy whereas the number of EPCs was increased [144]. Recently, a study showed that miR-21 overexpression impaired angiogenesis in ECs and also in a murine model of choroidal neovascularization by targeting the RhoGTPase Rhob [145]. According to this data, the overexpression of miR-21 decreased proliferation and impaired EPC angiogenesis by targeting high-mobility group A2 (Hmga2) *in vitro* and *in vivo* [146]. Interestingly, the endogenous nitric oxide synthase inhibitor, asymmetrical dimethylarginine (ADMA), which is increased in patients with CAD, was shown to induce the expression of miR-21 in angiogenic progenitor cells (APCs). This resulted in increased reactive oxygen species concentrations and impaired nitric oxide bioavailability through an indirect regulation of superoxide Dismutase 2 (SOD2) [147]. Additionally, high ADMA plasma levels and miR-21 expression are correlated in APCs from CAD patients, impairing their migration capacity. Conversely, inhibition of miR-21 in APCs from CAD patients recovered migratory capacity of APCs, suggesting that miR-21 antagonism might be a promising strategy to improve dysfunctional APCs in patients with coronary artery disease [147]. However, other studies reported a pro-angiogenic role of miR-21 in ECs [148] and tumors [149,150].

3.4. Endothelial cell senescence and microRNAs

EC senescence plays an important role in the pathogenesis of vascular dysfunction. Several miRNAs have been involved in this process such as miR-217 [151] and miR-146 [152] (Fig. 1A). In particular, Menghini et al. reported that miR-217 negatively regulated the expression of silent information regulator 1 (SirT1), a NAD^+-dependent deacetylase, which prevented stress-induced senescence and mediated angiogenesis through deacetylation of the forkhead transcription factor (FoxO1). Indeed, overexpression of miR-217 in young ECs induced a premature senescent-like phenotype and impaired angiogenesis whereas its inhibition in old ECs reduced senescence and increased angiogenesis [151]. On the other hand, aging, which is associated with an increase of reactive oxygen species (ROS) and promotes senescence, decreases the expression of miR-146 in ECs. In particular, Vasa-Nicotera et al. showed that miR-146 negatively regulated the expression of NOX4, the main endothelial isoform of the NADPH oxidases complex [152]. Therefore, overexpression of miR-146 might be a novel therapeutic tool for vascular disorders by targeting the production of ROS and therefore regulating senescence [152].

4. Control of leukocyte trafficking and inflammation by endothelial microRNAs

Inflammation is an integral part of a wide array of human diseases and it is an essential component of the innate immune response to pathogens and damaged cells. Inflammation is usually defined as the local recruitment and activation of leukocytes [10,14]. In response to pathogens or inflammatory cytokines including IL-1 and TNF-α, ECs get "activated" acquiring new capacities, therefore controlling a multi-step process in which leukocytes first transiently tether to and roll on the EC surface, then adhere more strongly and migrate over the endothelium, and finally squeeze between ECs to emigrate from the intravascular space to the extravascular sites of inflammation [153,154]. In resting conditions, leukocytes do not interact with ECs, because they sequester the proteins responsible for the interaction with leukocytes (*e.g.* P-selectin and chemokines) within secretory vesicles known as Weibel–Palade bodies [155,156]. In these conditions the expression of other adhesion molecules, such as E-selectin, vascular cell-adhesion molecule 1 (VCAM1) and intracellular adhesion molecule1 (ICAM1) is suppressed [157,158].

Recent studies have shown that inducible miRNAs also contribute to the regulation of inflammation [159] (Fig. 1B). Specifically, miR-155 and miR-146, as well as miR-132, miR-125b and miR-9, have been shown to be induced in different cell types (*e.g.* human lung alveolar epithelial cells, macrophages, neutrophils) by a variety of proinflammatory stimuli such as IL-1, TNF, IFN, LPS and Toll-like receptor (TLR) ligands [160–164]. In the context of ECs, in addition to miR-155 (prototype of TNF-induced miRNA in different cell types), TNF induces the expression of miR-31 and miR-17-3p, among others. Interestingly, E-selectin (E-SELE) and ICAM1 were identified and validated as targets of the TNF-induced miRNAs, miR-31 and miR-17-3p, respectively [165]. Specific antagonism of these TNF-induced miRNAs increased neutrophil adhesion to cultured ECs. Conversely, transfections with mimics of these miRNAs decreased neutrophil adhesion to ECs [165]. MiR-126, a constitutively expressed, but EC-restricted miRNA, has also been involved in vascular inflammation by modulating VCAM-1 expression induced by TNF [81]. However, the expression of this miRNA was affected by TNF treatment [165]. In response to TNF the expression of miR-181b has been shown to be reduced in ECs. MiR-181b regulates the expression of importin-α3 (KPNA4), a protein required for nuclear translocation of NF-kB. As activation of the transcription factor NF-kB is critical for TNF-induced inflammatory responses, miR-181b has been shown to indirectly decrease NF-kB responsive genes, such as VCAM1 and SELE in ECs *in vitro* and *in vivo* [166]. Importantly, the overexpression of miR-181b by intravenous delivery reduced the NF-kB pathway and decreased lung injury and mortality in endotoxemic mice while the endogenous inhibition of miR-181b increased the inflammatory phenotype. Consistent with this, the expression of miR-181b is reduced in patients critically ill with sepsis [166]. Altogether, these data suggest that the overexpression of miR-31, miR-17-3p, miR-126 and miR-181b could be an important tool for anti-inflammatory therapy in several pathological conditions including atherosclerosis and rheumatoid arthritis among others.

MiR-10a has been reported to negatively regulate the NF-kB pathway by targeting mitogen-activated kinase kinase kinase 7 (MAP3K7) and β-transducin repeat containing gene (βTRC), two regulators of the proteosomal degradation of IkBα and p65 translocation in ECs [167]. Interestingly, the expression of miR-10a is decreased in athero-susceptible arterial regions while the expression of MAP3K7 and βTRC is upregulated, suggesting that the differential expression of miR-10a could contribute to the regulation of pro-inflammatory endothelial phenotypes in athero-susceptible regions [167].

Angiotensin II (Ang II) can induce the expression of different adhesion molecules and therefore initiate the vascular inflammatory response. A recent study showed that miR-155 and miR-221/222 negatively regulated Ets-1 [132], a key transcription factor of endothelial inflammation and angiogenesis. Interestingly, in Ang II stimulated ECs, Ets-1 and several of its downstream genes include VCAM1, MCP1 and FLT1 were upregulated. This effect was partially reversed by overexpression of miR-155 or miR-221/222. In particular, the overexpression of miR-155 and miR-221/222 reduced leukocyte adhesion to ECs, as well as EC migration [132].

Oxidized Low Density Lipoproteins (Ox-LDL) are known to enhance the expression of pro-inflammatory genes, leading to monocyte recruitment into the vessel wall and dysfunction of vascular ECs [168]. MiR-125a/b are highly expressed in vascular ECs and their expression can be modulated by ox-LDL [169].

Ox-LDL-induced apoptosis in ECs is thought to play a critical role in atherosclerosis. Interestingly, miR-365 expression is regulated by ox-LDL in ECs and potentiates ox-LDL-mediated apoptosis by regulating the expression of Bcl-2 [170]. Atherosclerotic lesions preferentially originate and develop at arterial sites of curvatures, branches, and bifurcations where complex hemodynamic conditions of disturbed flow are associated with endothelial phenotypes expressing proinflammatory and procoagulant susceptibility [171]. The NF-κB signal transduction pathway has been shown to be primed for activation in regions predisposed to atherosclerotic lesion formation. Conversely, KLF2, KLF4, and nuclear factor erythroid 2–like 2 regulate gene networks that confer atheroprotective properties to the endothelium through an anti-inflammatory/antioxidant and anticoagulant phenotype profile. Fang et al. reported that the upregulation of miR-92a expression inversely correlated with KLF2 and KLF4 expression in atherosusceptible endothelium [172]. Indeed, miR-92 decreased KLF2 and KLF4 expression and knockdown of miR-92a partially suppressed TNF-induced endothelial inflammatory mediators through KLF4 and KLF2, therefore inhibiting TNF-induced leukocyte adhesion to ECs *in vitro* [172].

Gene expression profiles are dramatically altered when ECs are exposed to laminar shear (LS) or oscillatory shear (OS) stress. LS is known to increase expression of atheroprotective genes including KLF2, KLF4 and eNOS, while OS stimulates inflammation by overexpression of bone morphogenic protein-4 (BMP4) and adhesion molecules [171,173–175]. MiRNA microarray analysis of ECs exposed to OS or LS identified 21 miRNAs differentially expressed, including miR-663, which was found to be upregulated in OS. Treatment of ECs with the miR-663 antagonist blocked OS-induced monocyte adhesion, but not apoptosis. In contrast, overexpression of miR-663 increased monocyte adhesion in LS-exposed cells. Potential miR-663 targets included a network of inflammatory genes and transcription factors such as KLF4 [176].

5. Control of barrier function by endothelial microRNAs

ECs form a monolayer, within the blood vessel, by which the cells are linked to each other by different types of adhesive structures or cell-cell junctions (*i.e.* tight junctions, adherens junctions and gap junctions). This permits a selective barrier to form for the transport of molecules between blood and tissues [177,178]. These adhesive structures, which are involved in the control of the vascular permeability to circulating cells, also needs to be regulated in coordination with the functional requirements of the irrigated organ [179]. Changes in endothelial permeability are associated with redistribution of surface cadherins or occludins, stabilization of focal adhesion bonds and the progressive activation of matrix metalloproteases [180]. Loss of barrier function in pathophysiological situations can lead to extracellular edema. Different stimuli, such as histamine and thrombin, induce a rapid and short-lived increase in vascular permeability while others, like TNF or VEGF, induced a more sustained response. Of interest, most of these agonists are produced in situations of acute or chronic inflammation [181]. Recent work also indicates that miRNAs play an important role in controlling barrier function as described below and depicted in Fig. 1C.

MiR-125b expression has been shown to be transiently induced in ECs stimulated with VEGF. This miRNA inhibited the translation of vascular endothelial (VE)-cadherin mRNA. Because miR-125b induction in ECs is transient after VEGF stimulation, prolonged overexpression of miR-125b could result in blood vessel regression due to loss of normal barrier function [181].

Hantaviruses infect human ECs and cause two diseases marked by vascular permeability defects, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) [182]. Vascular permeability occurs in the absence of EC lysis, suggesting that hantaviruses alter normal EC fluid barrier functions [182]. Andes hantavirus (ANDV) infection of human ECs results in changes in the level of specific EC miRNAs, including miR-155, miR-320, and miR-222 [182]. These miRNAs reportedly regulate adherens junction disassembly, cell migration, and cell morphology, which contribute to changes in vascular permeability [61,62,183]. However, other miRNAs were downregulated 3–3400-fold following ANDV infection; these included miR-410, involved in regulating secretion, and miR-218, which is linked to the regulation of EC migration and vascular permeability [182]. In particular, this finding suggests that in ANDV-infected ECs decreased miR-218 levels may enhance VEGF-directed permeability by increasing Robo1 and thereby decreasing Robo4 regulation. Robo4 normally stabilizes the vasculature by counteracting VEGF signaling responses that result in EC hyperpermeability [184,185]. The changes in miR-126, an EC-specific miRNA that regulates vascular integrity by suppressing SPRED1 and PIK3R2 mRNAs [87,89], were also analyzed [182]. While miR-126 levels were only slightly altered, SPRED1 and PIK3R2 mRNA levels were increased 10- and 7-fold, respectively, in ANDV-infected ECs but were unaltered in ECs infected by the nonpathogenic Tula hantavirus. Consistent with increased SPRED1 expression, the level of phospho-cofilin was decreased within ANDV-infected ECs, therefore enhancing adherens junction disassembly responses [182].

6. Control of vascular tone by endothelial microRNAS

ECs control the secretion of two potent short-lived mediators that influence vascular hemodynamics in the physiological state and therefore contribute to the regulation of blood pressure and blood flow by releasing vasodilators, such as NO and prostacyclin or prostagladin I2 (PGI2), as well as vasoconstrictors, including ET and platelet activating factor (PAF) [4]. The specific blood-mediators released by ECs vary depending on the vascular bed, but the principal vasorelaxant is NO [9]. NO is generated in ECs by the oxidation of L-Arginine to L-Cytruline by eNOS [186,187]. eNOS is a constitutively expressed gene that can be upregulated by increases in shear stress of growth factors, such as VEGF and some drugs, including 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins). Conversely, hypoxia, lipopolysaccharide (LPS), TNF and ox-LDL decrease its expression [188]. In addition, eNOS function is also tightly regulated by its subcellular localization, protein-protein interactions and multi-site phosphorylation mostly by the protein kinases AKT and AMPK [186,189]. Regarding the role of miRNAs in regulating vascular tone (Fig. 1C), initial studies, showed that knockdown of Dicer, the enzyme necessary for miRNA maturation, increased eNOS expression in ECs [77]. Transfection of miR-221 and miR-222 mimics partially reversed the increases of eNOS protein attributable to Dicer silencing, suggesting miR-221 and miR-222 may be involved in the control of eNOS expression. However, the effect of these miRNAs on eNOS might be indirect since no predicted binding sites have been found in the eNOS 3′UTR [77]. Recent reports indeed provide evidence that miR-155 downregulated eNOS expression through decreasing eNOS mRNA stability by binding to its 3′-UTR [190]. Interestingly, TNF increased miR-155 expression in ECs [165] and knockdown of miR-155 prevented cytokine-induced downregulation of eNOS expression, reduction of NO production, and impairment of endothelium-dependent vascular relaxation [190]. Moreover, the authors found that simvastatin ameliorated TNF-induced endothelial dysfunction *via* inhibition of miR-155 expression [190].

NO has pleiotropic effects on the vasculature. It causes vascular smooth muscle cell (SMC) relaxation, which maintains

vasomotor tone, inhibits SMC migration and proliferation and prevents platelet and leukocyte activation and adhesion [4,191–193]. Failure of ECs to control these basal functions constitutes "EC dysfunction" leading to vessel constriction, thrombosis and inflammation. Local disturbances in blood flow trigger these undesired effects and explain why regions with disturbed flow, such as arterial branch points, have diminished NO secretion and are prone to develop atheromas [194]. Several reports showed that shear stress induced the expression of miR-21 with contrasting effects. In particular, laminar flow increased miR-21 and reduced apoptosis and increased nitric oxide (NO) production by modulating The PI3K/Akt pathway, overall ameliorating EC functions [195]. However, Zhou et al. reported that OS increased miR-21 expression, which repressed peroxisome proliferators-activated receptor-α (PPARα), a repressor of activator protein-1 (AP-1) signaling. In particular, AP-1 induced the expression of different adhesion molecules and miR-21 itself. These data showed that OS-induced miR-21 promoted endothelial inflammation [196]. These controversial findings might be explained by the difference in cellular context and, likely, also in the magnitude of upregulation of miR-21 induced by laminar or oscillatory shear stress.

In addition to producing the potent vasodilator NO, ECs also synthesize endothelin-1 (ET-1), one of the most potent vasoconstrictors [197]. ET-1 expression and secretion by ECs is stimulated by hypoxia, shear stress and ischemia. Upon release, it binds to the G-coupled ET-A receptor on vascular SMCs, resulting in an increase in calcium concentration and vascular SMC tone. NO controls the duration of these effects by accelerating the restoration of intracellular basal calcium levels. Therefore, in states of EC dysfunction, such as atherosclerosis, where NO levels are reduced, ET-1 promotes vasoconstriction and SMC proliferation [198]. MiR-125a/b has been involved in the regulation of the potent vasoconstrictor ET-1. Interestingly, an inverse correlation between the levels of miR-125a/b and the precursor protein ET-1 (preproET-1) has been reported in aortas in stroke-prone spontaneously hypertensive rats compared to normotensive rats [169]. Due to the relevance of ET-1 in many vascular diseases such as hypertension, atherosclerosis and stroke, targeting of miR-125a/b could provide an important therapeutic approach.

7. Control of endothelial cell functions by secreted microRNAs

MiRNAs are not only important as cell autonomous modulators of cellular functions, but also in light of their role as mediators of paracrine cell-to-cell communication [199]. Cells can communicate with other cells *via* multiple mechanisms. Besides passive protein and RNA shedding, cytokines, chemokines, enzymes or hormone secretion, they can release miRNA-protein complexes, lipoproteins containing miRNAs and vesicular particles that contain proteins and RNAs in the extracellular milieu [199].

Increasing evidence has demonstrated that many cell types are able to secrete extracellular microvesicles into the extracellular space that can be uptaken by other cells [200]. Since microvesicles retain surface receptors and ligands typical of their cells of origin, they might preferentially deliver their content to certain cell types. Because of the specificity of their content and of their potential targets, microvesicles can be both markers and mediators of disease [200].

The population of microvesicles is heterogeneous and includes vesicles from different origins. Although the classification of extracellular vesicles is still a matter of debate, it is well accepted to distinguish them based on the cellular process that they originate from [201].

Apoptotic bodies are large vesicles secreted by apoptotic cells and are heterogeneous in size and composition. Usually apoptotic bodies are identified as vesicles of 50–500 nm in diameter [202]. Shedding microvesicles have a diameter of 100–1000 nm and are produced by budding from the extracellular membrane and contain proteins and nucleic acids. Exosomes are around 30–100 nm in diameter and are produced within the endosomal compartment into multivesicular bodies. Exosomes are identified by the presence of tetraspanins (CD63, CD9, CD81) as well as endosomal proteins, such as Alix and Tsg101. They contain RNAs, especially miRNAs, which can be delivered to target cells. Interestingly, exosome protein and RNA composition depends on the activation status of the cell of origin [203], but it does not mirror its composition, as some miRNAs are specifically enriched in exosomes and not in cells. Exosomal proteins might be able to target exosomes to specific cells that have cognate membrane proteins [204].

ECs can both produce extracellular vesicles that target other cells and modify their function, or they can be targets of extracellular vesicles derived from other cells (Fig. 1D).

Human monocytes can secrete microvesicles containing miRNAs that are uptaken by ECs and increase their migratory capabilities. MiR-150 is contained in monocyte-derived microvesicles, both from the monocytic cell line, THP1, and from human peripheral blood monocytes [205]. The miRNA can be transferred to ECs and induce downregulation of c-Myb, which is responsible for the increased migratory capabilities of ECs. Moreover, patients with severe atherosclerosis had circulating microvesicles that were enriched in miR-150 compared to control patients, suggesting that their microvesicles might be either partially responsible for the vascularization of atherosclerotic plaques or might be a marker of it, since they have a higher monocyte infiltration in the lesion [205].

Tumor cells have been shown to produce large amounts of microvesicles. Several reports show that microvesicles have a positive effect on tumor development, by targeting, immune cells and ECs, among others [206,207].

Recent works have provided evidence that cancer-derived microvesicles can regulate EC functions. Cancer stem cells (SCs) have the capacity to initiate tumors from single cells. They are $CD105^{+}$ cells and can give rise to cells of multiple lineages, like epithelial cells and ECs. Cancer SCs can produce microvesicles that are characterized by the surface expression marker CD105 and, have the ability to prepare the microenvironment to promote tumor growth and angiogenesis. In particular, CD105 microvesicles display enrichment in certain miRNAs, whose main targets are involved in crucial biological processes and likely angiogenesis [207].

Zhuang et al. described that the ability of different tumor cell lines (lung cancer, pancreatic cancer, melanoma, glioblastoma, colorectal cancer) to modify EC functions is through microvesicle-derived miRNAs. Among the miRNAs transferred by tumors through microvesicles, they identified miR-9, which activates the JAK/STAT pathway by downregulating suppressor of cytokine signaling 5 (SOCS5) to induce EC migration [208].

Apoptotic bodies produced by ECs during atherosclerosis contain miRNA-126. By targeting the regulator of G protein signaling 16 (Rgs16), an inhibitor of G protein-coupled receptor signaling (GPCR), miR-126 increases the expression of chemokine receptor type 4 (CXCR4), which in turn increases chemokine receptor type 12 (CXCL12) that counteracts apoptosis and recruits progenitor cells in mice with atherosclerosis [209].

EPCs are believed to exert a protective effect in many disease models. It is well accepted that EPCs can secrete paracrine protective factors, but recent studies have shed light on the important role of EPC-derived microvesicles and their miRNA cargo. In fact, the regenerative effects of EPCs in a model of ischemia-reperfusion injury in the kidney as well as hind limb ischemia have also been attributed to EPCs ability to secrete miRNA-containing

microvesicles, and specifically miR-126 and miR-296-rich vesicles [210,211]. Those microvesicles could improve capillary rarefaction, glomerulosclerosis and tubulointerstitial fibrosis in kidney injury and increase capillary density in ischemia. Importantly, the ability of microvesicles to produce a phenotype was lost after treatment with RNase or if they were deprived of miRNAs by silencing of Dicer [210,211].

Upon shear stress, ECs can secrete microvesicles that are able to mediate an atheroprotective effect on vascular SMCs. Hergenreider et al. induced the production of microvesicles from ECs overexpressing the shear-responsive gene KLF2 or under shear stress. Those microvesicles were enriched in miRNAs, particularly miR-143/145, that mediated an atheroprotective action on smooth muscle cells [212]. Similarly, delivery of miR-143/145-enriched vesicles in ApoE$^{-/-}$ mice fed a high fat diet induced an atheroprotective effect, which was reverted if the microvesicles were depleted of miR-143/145.

Several studies also identified miRNAs in plasma or serum as biomarkers in certain diseases associated with EC function (Fig. 1D). Blood miRNAs are extremely stable to freeze/thaw cycles and RNAse treatment, raising the possibility that these miRNAs could be derived from circulating microvesicles produced by damaged cells [213,214].

Fichtlscherer et al. identified miRNAs dysregulated in patients with coronary artery disease [215]. In particular they found that miR-126, miR-155, and miR-145 were significantly reduced in patients with CAD and diabetes, while miR-17 and miR-92a were downregulated only in CAD patients. However, the origin and functional significance of these circulating miRNAs needs to be further explored.

Type 2 diabetes critically affects some EC functions and impairs postischemic reparative neovascularization. Therefore the study of miRNAs that are both markers and potential therapeutic treatments is of pivotal importance. Zampetaki et al. analyzed microvesicles from patients with type 2 diabetes and confirmed the downregulation of miR-126, as well as of other miRNAs, and among them miR-15a, miR-29b, miR-223, which were associated with an incidence of diabetes mellitus over 10-years [216]. Interestingly, miR-126 abundance in microvesicles produced by ECs treated with high glucose was also decreased compared to controls, suggesting an endothelial origin of the miR-126-depleted microvesicles in diabetic patients that also contribute to EC dysfunction.

In patients with diabetes-induced critical limb ischemia, a miRNA critical in EC function was found to be upregulated in the plasma of patients compared to controls, as well as in their muscles. High D-glucose treatment of ECs, which mimics diabetic conditions, induces the increase in expression of miR-503. This miRNA in turn decreases the angiogenic properties of ECs through repression of cdc25A and CCNE1 (as described above) [141]. Moreover, the anti-angiogenic miR-15 and miR-16 were found to be upregulated in patients with critical limb ischemia, without correlations with type 2 diabetes, further suggesting that circulating miRNAs in diabetes might promote the anti-angiogenic phenotype of ECs [138].

A note of caution should be addressed on the issue of detecting secreted miRNAs in patients. It is well known that diseases profoundly change the miRNA expression in cells. Furthermore, pathologies change the rate of release of miRNAs from different tissues, as well as their stability, profoundly affecting the abundance of miRNAs or other RNAs that are normally used as controls in healthy patients. Therefore common qPCR techniques that involve the normalization of the target miRNA relative expression with control-unchanged miRNAs should be analyzed carefully. Similarly, lack of a normalization control is also an issue for the quantification of microvesicle-derived miRNAs.

8. Pharmacological potential of microRNAs for endothelial cell dysfunction

Tissue specificity and the capability of coherently targeting multiple pathways are features that make miRNAs research targets of valuable therapeutic interest.

MiRNAs could be inhibited, exploiting chemically stable complimentary oligonucleotides [217], or their activity mimicked with so-called mimics [218]. Inhibition of miRNAs might be achieved by chemically stabilized 2′-O-methyl oligonucleotides (or anti-miRs) complimentary to the miRNA of interest [217]. Anti-miRs were first shown to be effective in *Drosophila melanogaster* and *Caenorhabditis elegans*, where they were used to inhibit let-7 [217]. It is believed that anti-miRs irreversibly bind to the complementary target miRNA and block their processing by the miRISC complex, however the mechanism has not been fully investigated [218,219].

Linking 2′-O-methyl anti-miRs with cholesterol, a process to generate the so-called antago-miRs, increases the cellular uptake and bioavailability in larger animals and their action has been shown to last up to 23 days when delivered by tail vein injection in mice and target most tissues [220]. The effects of anti-miRs and antago-miRs have mostly been elucidated in rodents and only a few reports have extended their studies on animal models closer to humans. For example, miR-33 inhibition by anti-miR in non-human primate African green monkeys was reported to be effective in increasing HDL plasma levels by upregulation of hepatic ABCA1 [221]. In addition to anti-miRs and antagomiRs, LNA-modified phosphorothioate oligonucleotide DNAs have been developed to inhibit miRNAs *in vitro*, as well as *in vivo* [222,223]. Of note, the therapeutic long-lasting efficacy of LNA-anti-miRs against the liver-specific miR-122 has been demonstrated in African green monkeys, as well as chimpanzees, to decrease plasma cholesterol levels and to prevent replication of the hepatitis C virus (HCV) [224,225]. A clinical trial of LNA-anti-miR-122, called miravirsen, on HCV patients is now in Phase IIa and has so far established the safety, as well the efficacy, of miravirsen in inhibiting HCV disease progression.

Delivery of a high number of miRNA target sequences that decoy binding sites and reduce the availability of the target miRNA, a strategy called ‘sponging’, has proven to be effective in knocking down the functionality of miRNAs. Successful sponging of miRNAs *in vivo* was first reported in bone marrow derived cells that were transduced with lentiviral particle encoding for miR-223 targets and could reconstitute the hematopoietic system of receiving mice [226].

Even though sponging of miRNAs has been successful in mouse models, it has some limitation due to the nature of genetic material transferred, usually bulky plasmids delivered by viral vectors [227]. On the other hand, oligonucleotide-based inhibition is more promising in the light of potential future chemical improvements that might generate more readily delivered, targeted and long-lasting oligonucleotide inhibitors.

The opposite strategy of mimicking miRNA function has been proven to be effective *in vivo* in various experimental conditions. For example, delivery of miR-16 mimics, which is an anti-oncogenic and anti-angiogenic miRNA as discussed above, suppresses the progression of tumors [228]. Even though studies of miRNA inhibition and overexpression *in vivo* do not report any long-lasting side effect, it should be kept in mind that miRNA are able to target multiple pathways and generate important off-target effects that could profoundly affect vital cellular functions. Additionally, miRNA targets are not always conserved among species and therefore off-target effects that are not present in other animals might represent a significant problem in humans. Therefore the action of therapeutically interestingly miRNAs has to be extensively investigated *in vitro* and *in vivo*, not only in rodents but also in higher-order animals like non-human primates, to confirm the specificity of

miRNA interaction with its targets and exclude side effects. Overall, the successful studies in rodents and non-human primates, as well as the preliminary results on clinical trials, show that miRNA inhibition and overexpression is a promising therapeutic tool in diseases and hopefully it will pave the way to human clinical trials of angiogenic/anti-angiogenic miRNAs to cure vascular diseases.

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

Acknowledgements

We thank Leigh Goedeke for the editing work on this review manuscript. YS Laboratory is supported by a grant from the National Institutes of Health R01HL105945. EA is a Howard Hughes Medical Institute International Student Research Fellow.

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